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Hydrolysis and Synthesis of ATP by Membrane-Bound ATPase from a Motile *Streptococcus*

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Abstract. ATPase was detected in the membranes of a motile *Streptococcus*. Maximal enzymic activity was observed at pH 8 and ATP/Mg^{2+} ratio of 2. Mn^{2+} and Ca^{2+} could replace Mg^{2+} to some extent. Besides ATP, GTP and ITP were substrates. The enzyme was inhibited by N,N′-dicyclohexylcarbodiimide but not by sodium azide, uncouplers or bathophenanthroline.

An electrochemical gradient of protons, which was artificially imposed across the membranes of *Streptococcus* cells by manipulation of either the K^+ diffusion potential or the transmembrane pH gradient, led to ATP synthesis. ATP synthesis was abolished by proton conductors, an inhibitor of the ATPase or an increase in the extracellular K^+ concentration. A comparison between the phosphate potential and the electrochemical proton gradient showed that the data found are in agreement with a stoichiometry of 2 protons translocated per molecule ATP synthesized.

Key words: Motile *Streptococcus* – Membrane-bound ATPase – DCCD – Valinomycin – Uncouplers – Protonmotive force – ATP synthesis.

Energization of various membrane-bound processes, such as active transport of neutral and ionic solutes and ATP synthesis via the membrane-bound proton-translocating ATPase (adenosine phosphohydrolase, EC 3.6.1.3), is brought about by chemiosmotic coupling (Mitchell, 1966; Harold, 1972; Hamilton, 1975; Haddock and Jones, 1977). In aerobic and facultatively anaerobic organisms the driving force for these processes – the so-called protonmotive force or electrochemical proton gradient ($\Delta\mu_{H^+}$) – is generated by the redox reactions of the electron transport chain. Anaerobic fermentative and facultatively anaerobic organisms in the absence of oxygen generate a $\Delta\mu_{H^+}$ via an electrogenic proton-translocating ATPase. The $\Delta\mu_{H^+}$ is composed of an electrical and a chemical parameter according to the relationship $\Delta\mu_{H^+} = \Delta\psi - Z\Delta$pH, were $\Delta\psi$ represents the membrane potential and ΔpH the transmembrane pH difference. Z is equal to 2.3 RT/F and amounts to 59 mV at 25° C.

Recently it was shown that an electrochemical gradient of protons is involved in motility and chemotaxis. Proton circulation not ATP delivers the energy necessary to drive the flagellar motor (Larsen et al., 1974; Manson et al., 1977) and the transmittance of a signal from a chemoreceptor to the flagellar motor is thought to occur via fluctuations in the $\Delta\mu_{H^+}$ or in the flux of a particular ion (de Jong et al., 1976; Szmelcman and Adler, 1976; Manson et al., 1977; Matsuura et al., 1977; de Jong and van der Drift, 1978). Previously the motile and chemotactic behavior of a motile *Streptococcus* was studied (van der Drift et al., 1975; Manson et al., 1977). Since this *Streptococcus* is a primarily fermentative organism, $\Delta\mu_{H^+}$ will be generated mainly via the membrane-bound ATPase. In this study we report on the properties of the membrane-bound ATPase of this motile *Streptococcus*. Both hydrolysis of ATP and synthesis of ATP under influence of an artificially imposed $\Delta\mu_{H^+}$ was studied. ATP synthesis in bacterial systems by means of an artificially imposed $\Delta\mu_{H^+}$ previously was shown for whole cells of *Escherichia coli* (Grinius et al., 1975), *Streptococcus lactis* (Maloney and Wilson, 1975) and *Halobacterium halobium* (Danon and Caplan, 1976), membrane vesicles of *E. coli* (Tsuchiya and Rosen,

Abbreviations. $\Delta\mu_{H^+}$ = electrochemical gradient of protons; DMO = 5,5-dimethyl-2,4-oxazolidinedione; CCCP = carbonylcyanide *m*-chlorophenylhydrazone; FCCP = carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DCCD = N,N′-dicyclohexylcarbodiimide; DNP = 2,4-dinitrophenol

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1976) and a thermophilic bacterium (Sone et al., 1977), and chromatophores of *Rhodospirillum rubrum* (Gromet-Elhanan and Leiser, 1975).

Materials and Methods

Microorganism. The motile *Streptococcus* Strain V4051 (van der Drift et al., 1975) was used in all experiments.

Media, Growth Conditions and Preparation of Cell Suspensions. The strain was grown at 37° C in 3.7% brain heart infusion broth (Oxoid) supplemented with 0.5% KCl. Cells were harvested by centrifugation (8,000 × g for 10 min at 4° C) in the early stationary growth phase at a cell density of 0.5 mg of dry weight per ml. The cells were washed twice with 100 mM sodium phosphate buffer (pH 7.0) and resuspended in this buffer to 0.4–0.6 mg of dry weight per ml.

Preparation of Membranes. Cells were harvested by centrifugation and washed twice with 100 mM Tris-HCl buffer (pH 8.0) containing 2.5 mM $MgCl_2$. The cells were resuspended in this buffer (5 ml buffer per g wet weight) and lysed by passage through a French pressure cell (American Instrument Co.) at 140,000 kPa. The suspension was centrifuged at 20,000 × g for 10 min and the supernatant solution was centrifuged at 100,000 × g for 1 h at 4° C. The membranes were washed and resuspended in Tris-Mg^{2+} buffer (pH 8.0) (about 5 mg of protein per ml), frozen in liquid nitrogen and stored at −20°C.

Assays. The ATPase activity of the isolated membranes was assayed by measurement of inorganic phosphate (Bonting et al., 1961) liberated from ATP at 37° C. The incubation mixture routinely contained 100 mM Tris-HCl (pH 8.0), 5 mM ATP, 2.5 mM $MgCl_2$ and a suitable amount of membrane protein (200–600 µg per ml). One unit of activity is defined as the amount of enzyme which liberates 1 µmol inorganic phosphate per min at pH 8.0 and 37° C. Specific activity is expressed as units per mg of protein.

Intracellular ATP was extracted as described by Maloney and Wilson (1975). The extracted ATP was measured according to Cole et al. (1967). ADP was measured after its conversion to ATP in the presence of pyruvate kinase and excess phosphoenolpyruvate (Chapman et al., 1971). Intracellular inorganic phosphate was extracted by treatment of the cells for 15 min at 0° C with 1 N $HClO_4$ (10 mg of dry weight per ml $HClO_4$). After neutralization with KOH and removal of cells and precipitate by centrifugation inorganic phosphate was measured (Bonting et al., 1961).

Intracellular potassium was determined by flame photometry (Maloney and Wilson, 1975).

The intracellular pH was calculated from the distribution of the weak acid [^{14}C]-5,5-dimethyl-2,4-oxazolidinedione (DMO) between the intracellular space of the cells and the medium using [^{3}H]-inulin as marker for extracellular water (Rottenberg, 1975).

Cell water was determined with the use of $^{3}H_2O$ and [^{14}C]-inulin assuming that water freely permeates the cell membrane, whereas inulin does not.

Protein was measured according to Lowry et al. (1951) using bovine serum albumin as standard.

Proton Conduction. The rate of H^+ entry at 24° C was measured according to Harold and Baarda (1968).

ATP Synthesis. Washed cells were resuspended (0.4–0.6 mg dry weight per ml) in 100 mM sodium phosphate buffer of the desired pH. An artificial $\Delta\mu_{H^+}$ was applied at 24°C either by addition of the potassium ionophore valinomycin, a pH shift or a combination of both. At different time intervals samples were taken and assayed for ATP content. The size of the $\Delta\mu_{H+}$ was calculated from the contributions made by both the membrane potential and the pH gradient. The membrane potential was determined from the measured ratio of internal to external potassium using the Nernst equation. The activity coefficient for K^+ was assumed to be 1 both for the internal space and the extracellular medium. The pH gradient was determined from measurements of internal and external pH and expressed in terms of millivolts (59 ΔpH).

The phosphate potential – the free energy of synthesis of ATP – was calculated from the intracellular concentrations of ATP, ADP and inorganic phosphate and using a value of 28,5 kJ/mol for the standard free energy of hydrolysis of ATP at pH 7.0, 10 mM Mg^{2+}, 0.2 ionic strength and 25° C (Rosing and Slater, 1972). Since the intracellular inorganic phosphate concentration (37 mM) was much higher than the nucleotide concentration, changes in this concentration were neglected.

Chemicals. ATP, ADP, ITP, GTP and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were obtained from Boehringer, Mannheim; valinomycin and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) from Calbiochem, San Diego, CA; N,N′-dicyclohexylcarbodiimide (DCCD) from Britisch Drug Houses, Poole; bathophenanthroline from Merck, Darmstadt; firefly lantern extract (FLE-50) from Sigma, St. Louis, MI; [^{3}H]-inulin from The Radio Chemical Centre, Amersham; and [^{14}C]-5,5-dimethyl-2,4-oxazolidinedione (DMO) and $^{3}H_2O$ from New England Nuclear Corp., Boston, MA. Solutions of valinomycin, CCCP and DCCD were made in methanol.

Results

Proton Conduction

Cells took up protons slowly when the pH of a lightly buffered cell suspension was lowered from 6.5 to 5.3. Addition of valinomycin resulted in an increase of H^+ uptake and subsequent addition of either CCCP or DNP permitted a very rapid entry of H^+ into the cells (Fig. 1). Pretreatment of the cells for 15 min at 24° C with DCCD (10^{-4}M) resulted after acidification of the cell suspension in a H^+ uptake which was about half that of untreated cells. After addition of valinomycin to DCCD- treated cells a light increase in H^+ uptake was observed, while addition of uncoupler resulted in a very rapid H^+ uptake (Fig. 1).

Hydrolysis of ATP

Cells of *Streptococcus* strain V4051 were fairly resistant to lysozyme even after acetylation of the cell wall as described by Eisenberg and Lillmars (1975). Therefore membranes were prepared by using a French pressure cell. The specific ATPase activity of such membranes amounted to 0.3. Membranes with a similar specific activity were obtained if the cells were broken by ultrasonic disintegration. The streptococcal membrane-bound ATPase showed optimal activity at pH 8.0. The enzyme required Mg^{2+} for activity and the reaction velocity was maximal at an ATP/Mg^{2+} ratio of 2. Mg^{2+} could be partially replaced by Mn^{2+} and Ca^{2+}: in the case 80% and 35%, respectively, of the activity with Mg^{2+} was measured. The enzyme exhibited Michaelis-Menten kinetics with a K_m value for ATP of 1.8 mM. GTP, ITP and ADP were hydrolyzed by the enzyme at a rate which was 100%, 70% and less

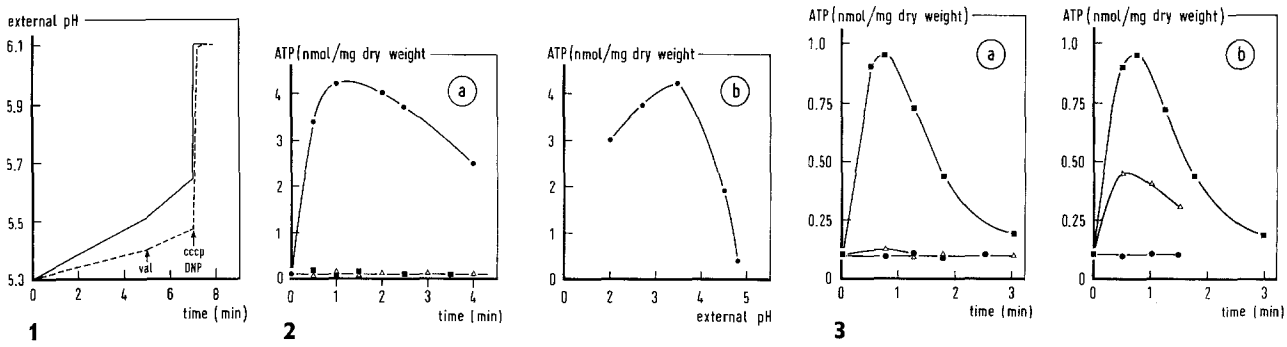

Fig. 1. Proton uptake by *Streptococcus* strain V4051. Cells were suspended (0.21 mg dry weight/ml) at 24°C in KCl (50 mM)-$MgCl_2$ (2 mM)-glycylglycine (1 mM). To 10-ml portions (initial pH 6.5) 0.05 ml 10 mM HCl was added followed by further additions as indicated by the *arrows*. The *dashed line* represents cells treated for 15 min at 24°C with DCCD (10^{-4} M) before the addition of HCl. Concentrations used were: valinomycin (*val*), 10^{6} M; CCCP, 10^{-5} M; DNP, 5×10^{-4} M

Fig. 2a and b. ATP synthesis driven by a pH gradient. Cells (0.53 mg dry weight/ml) were suspended in 100 mM sodium phosphate (pH 8.0). A pH gradient was applied by addition of various amounts of HCl. ATP levels were determined as described in "Materials and Methods". **a** Time-dependent ATP synthesis after a pH shift from 8 to 3.5. ●——● none addition; ■——■ cells preincubated for 15 min with 10^{-4} M DCCD; △——△ cells preincubated for 1 min with 10^{-5} M CCCP. **b** ATP synthesis as function of the pH gradient. The maximal level of ATP was determined for all shifts applied in external pH. All experiments were carried out at 24° C

Fig. 3a and b. ATP synthesis driven by a combination of a membrane potential and a pH gradient. Cells (0.5 mg dry weight/ml) were suspended in 100 mM sodium phosphate buffer (pH 7.0). An $\Delta\mu_{H^+}$ was imposed by addition of HCl, valinomycin or a combination of both. ATP levels were determined as described in "Materials and Methods". **a** Time-dependent ATP synthesis. △——△ valinomycin (10^{-5} M); ●——● the external pH was shifted from 7 to 5.2; ■——■ the external pH was shifted to 5.2 and valinomycin (10^{-5} M) was added. **b** Effect of external K^+ on ATP synthesis. ■——■ both the external pH was shifted to 5.2 and valinomycin (10^{-5} M) was added, external K^+ 0.3 mM; △——△ external K^+ 1.3 mM; ●——● external K^+ 10.3 mM

than 2%, respectively, of that observed with ATP as substrate. The enzyme was inhibited by DCCD: preincubation for 15 min at 24° C with DCCD (2×10^{-4}M) lowered the ATPase activity about 70%. No inhibition was observed with sodium azide (100 mM), CCCP (0.05 mM), FCCP (0.01 mM), DNP (1 mM) and bathophenanthroline (1 mM). Preincubation of the membrane-bound ATPase for 10 min at 37°C with trypsin (0.5 mg/ml) lowered the activity to 75%.

No effective and selective solubilization of the membrane-bound enzyme was obtained when the membranes were either washed with buffers of low ionic strength in the absence of Mg^{2+} (Abrams, 1965) or treated with NH_4HCO_3 and EDTA at pH 9 (Carreira et al., 1973).

ATP Synthesis Driven by a pH Gradient

The ATP level in nonstarved and starved cells of *Streptococcus* strain V4051 is about the same (about 100 pmol/mg dry weight). The intracellular pH of the cells was 6.8 as measured by [^{14}C]-DMO distribution. This value is in agreement with that determined by Manson et al. (1977) after lysis of the cells in n-butanol. The establishment of an inwardly directed $\Delta\mu_{H^+}$ by imposing a pH gradient (alkaline inside) across the cell membrane resulted in ATP synthesis. At pH 3.5 the ATP level increased maximally about 40-fold. No ATP synthesis after a pH jump was observed if CCCP, a proton conductor, or DCCD, an ATPase inhibitor, was present (Fig. 2a). The magnitude of the applied pH gradient determined the maximal increase in the ATP level. A transition from pH 8 to pH 3.5 was optimal for ATP synthesis, while a shift from pH 8 to pH 5 did not result in ATP formation (Fig. 2b).

ATP synthesis was dependent on the growth phase of the cells. It appeared that maximal ATP synthesis occurred in cells from the early stationary phase. Therefore such cells were used in all experiments.

ATP Synthesis Driven by a Membrane Potential and a pH Gradient

Cells harvested in the early stationary phase have a K^+ content of 600 mM. The K^+ content of washed cells used in our experiments was 220 mM. Addition of valinomycin (10^{-5}M) to cells suspended in potassium-free sodium phosphate buffer (pH 7.0) resulted in the synthesis of a very small amount of ATP (see also Manson et al., 1977). If both an artificially imposed membrane potential and pH gradient were applied a considerable amount of ATP was synthesized; the pH gradient by itself was insufficient to induce ATP synthesis (Fig. 3). ATP synthesis was abolished by

Table 1. Correlation between ATP synthesis and the electrochemical proton gradient. Cells (0.5 mg dry weight/ml) were suspended in 100 mM sodium phosphate buffer (pH 6.8) with different concentrations of K^+. An $\Delta\mu_{H^+}$ was applied by addition of valinomycin (10^{-5}M) and/or HCl. ATP synthesis was measured as described in "Materials and Methods". The size of the $\Delta\mu_{H^+}$ was calculated from the contributions of the pH gradient and the membrane potential

Maximal ATP synthesis (nmol/mg dry weight)	$\Delta\psi$ (mV)	$-Z\Delta$pH (mV)	$\Delta\mu_{H+}$ (mV)
0.1	104	0	104
0.5	137	8	141
0.7	104	67	171
0.9	104	72	176
0.9	137	40	177
1.0	137	41	178
1.0	104	76	180
1.4	137	60	197
1.5	137	66	203
1.3	104	168	272
1.2	137	204	341

Table 2. Correlation between phosphate potential and electrochemical proton gradient. Experimental conditions are given in Table 1. The phosphate potential and the $\Delta\mu_{H^+}$ were measured as described in "Material and Methods"

Phosphate potential		$\Delta\mu_{H^+}$ (mV)	
ATP/ADP	$\Delta G'$ (kJ/mol)	$\Delta\mu_{H^+}$ required for $\Delta G'$ assuming $2H^+$/ATP	$\Delta\mu_{H^+}$ applied
0.3	33.7	174	161
1.6	37.8	196	176
2.5	38.9	202	197
2.7	39.1	202	187
3.1	39.5	205	187
4.7	40.5	209	176
6.3	41.2	213	198

either CCCP or DCCD (data not shown). Addition of KCl to the external medium reduced ATP synthesis as would be expected since the driving force for ATP synthesis was mainly composed of a K^+ diffusion potential.

Relation between ATP Synthesis and Electrochemical Proton Gradient

Table 1 shows the correlation between ATP synthesis and the applied $\Delta\mu_{H^+}$. At all values of $\Delta\mu_{H^+}$ net ATP synthesis was observed. Maximal synthesis seemed to be reached at about 200 mV, while below 140 mV only a slight increase in ATP levels was observed.

Comparison of Phosphate Potential and Electrochemical Proton Gradient

Table 2 shows that the phosphate potential measured under the experimental conditions used would require an $\Delta\mu_{H^+}$ of about 200 mV if a stoichiometry of 2 protons translocated per ATP synthesized is assumed. The values of $\Delta\mu_{H^+}$, which were measured simultaneously, were in reasonable agreement with the phosphate potential based on the above-mentioned assumption.

Discussion

The properties of the membrane-bound ATPase of *Streptococcus* strain V4051 resemble those of other membrane-bound bacterial ATPases (Abrams and Smith, 1974; Panet and Sanadi, 1976). The enzyme shows maximal activity at pH 8.0 and a ATP/Mg^{2+} ratio of 2. Mg^{2+} can be partially replaced by Mn^{2+} and Ca^{2+} ions. Besides ATP, GTP and ITP are substrates. The enzyme is inhibited by DCCD, but not by sodium azide, CCCP, FCCP and DNP. Bathophenanthroline, a lipophilic chelator, which inhibits the *Escherichia coli* ATPase (Sun et al., 1975) has no effect on the streptococcal enzyme. Trypsin, which stimulates the ATPase activity of various bacteria (Carreira et al., 1973; Panet and Sanadi, 1976) does not activate the streptococcal ATPase, but inactivates it just as was observed with the enzyme from *Clostridium pasteurianum* (Riebeling and Jungermann, 1976). The streptococcal ATPase remains membrane-bound when washing procedures are employed using dilute buffers in the absence of Mg^{2+} ions (Abrams, 1965; Carreira et al., 1973; Abrams and Smith, 1974).

An artificially imposed electrochemical gradient of protons either brought about by a pH gradient or a pH gradient in combination with valinomycin results in a transient synthesis of ATP. This synthesis does not take place in the presence of a proton conductor or an inhibitor of the ATPase and furthermore is dependent on the external K^+ concentration. Cells pretreated with DCCD show a much lower proton uptake than untreated cells indicating the role of the ATPase in proton translocation. These observations can be fully explained by the chemiosmotic hypothesis (Mitchell, 1966; Harold, 1972) and are in agreement with observations made with other bacteria (Grinius et al., 1975; Maloney and Wilson, 1975; Danon and Caplan, 1976).

It was proposed that 2 protons are translocated per ATP molecule hydrolyzed or synthesized (Moyle and Mitchell, 1973). A comparison of the $\Delta\mu_{H^+}$ values generated artificially across the cell membrane of *Streptococcus* strain V4051 with the values of the

phosphate potential assuming a H^+/ATP ratio of 2 shows that the values are in reasonable agreement. A translocation of 3 protons per ATP synthesized was observed with mitochondria (Nicholls, 1974), chloroplasts (Avron, 1976) and chromatophores of *Rhodospirillum rubrum* (Leiser and Gromet-Elhanan, 1977). However, in chromatophores of *Rhodopseudomonas capsulata* the measured $\Delta\mu_{H^+}$ values were identical to or in excess of those required by the measured phosphate potential assuming a H^+/ATP ratio of 2 (Casadio et al., 1974). Recently it was proposed that in mitochondria 3 protons are ejected per pair of electrons traversing each energy- conserving site of the respiratory chain and that 2 protons return to the cytoplasm through the ATPase to from ATP from ADP and phosphate or alternatively that 4 protons are ejected per site followed by return of 3 protons through the ATPase (Brand and Lehninger, 1977)

Our data indicate that in *Streptococcus* strain V4051 the H^+/ATP ratio is 2. However, this ratio may vary with different organisms and further work will be needed to elucidate this.

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